CHARACTERIZATION OF A SHIGA-LIKE TOXIN CONVERTING PHAGE ISOLATED FROM AN ESCHERICHIA COLI STRAIN RESPONSIBLE FOR HEMORRHAGIC COLITIS IN HUMANS

by

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Dissertation submitted to
the Faculty of the Department of Microbiology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirement for the degree of
Master of Science 1985

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER: AFIT/CI/NR 85-84T	2. GOVT ACCESSION NO.: AD-A157 955
3. RECIPIENT'S CATALOG NUMBER:	
4. TITLE (and Subtitle): Characterization of a Shiga-Like Toxin Converting Phage Isolated from an Escherichia coli Strain Responsible for Hemorrhagic Colitis in Humans	5. TYPE OF REPORT & PERIOD COVERED: THESIS/DISSERTATION
	6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): Steven Franklin Miller	8. CONTRACT OR GRANT NUMBER(s):
9. PERFORMING ORGANIZATION NAME AND ADDRESS: AFIT STUDENT AT: Uniformed Services University of the Health Sciences	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS:
11. CONTROLLING OFFICE NAME AND ADDRESS: AFIT/NR WPAFB OH 45433	12. REPORT DATE: 1985
	13. NUMBER OF PAGES: 70
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): UNCLASS
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE:

16. DISTRIBUTION STATEMENT (of this Report)

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

APPROVED FOR PUBLIC RELEASE: IAW AFR 190-1

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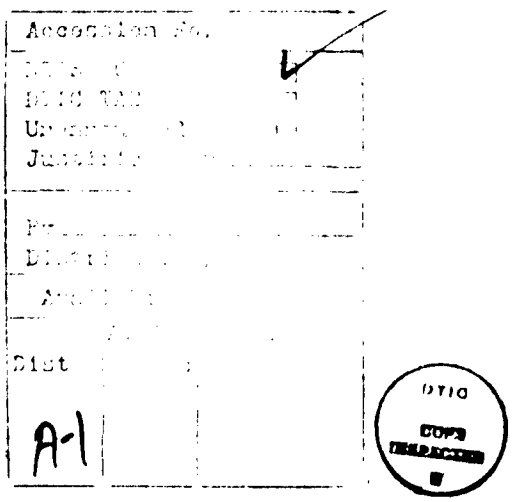

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of the Health Sciences

ABSTRACT

Title of dissertation: Characterization of a Shiga-like Toxin Converting Phage Isolated from an Escherichia coli Strain Responsible for Hemorrhagic Colitis in Humans

Steven Franklin Miller, B.S.; Candidate, Master of Science, 1985

Dissertation directed by: Alison D. O'Brien, Ph.D., Associate Professor, Department of Microbiology

There is a strong correlation between the ability of Escherichia coli to produce high levels of Shiga-like toxin in vitro and to cause hemorrhagic colitis in humans. A Shiga-like toxin-converting phage from Escherichia coli 0157:H7 strain 933 (phage 933J) and another Shiga-like toxin-converting phage from Escherichia coli 026 strain H-19 (phage H-19J) were isolated and found to be closely related by morphology, virion polypeptide composition, pattern of DNA restriction fragments, heat stability, and lysogenic immunity. However, a difference was noted between phage 933J and H-19J in the range of bacterial hosts on which these phages would plaque. The phage 933J toxin-converting genes were cloned into pBR328 and expressed in Escherichia coli HB101. DNA restriction mapping, subcloning, and examination of the cloned gene products by minicell analysis were used to localize the toxin-converting genes and identify them as the structural genes for Shiga-like toxin. Southern hybridization studies demonstrated that the DNA fragment containing the cloned toxin structural genes was homologous with the chromosome of Shigella.

Note: p. iv is title page.

DEDICATION

I dedicate this thesis to the pilots (past, present, and future) of the United States Air Force who have valiantly defended our country during crises and peacetime. As John Gillespie Magee, Jr. so eloquently stated in the poem "High Flight:"

Oh, I have slipped the surly bonds of earth
And danced the skies on laughter-silvered wings;
Sunward I've climbed and joined the tumbling mirth
Of sun-split clouds——and done a hundred things
You have not dreamed of——wheeled and soared and swung
High in the sunlit silence. Hovering there
I've chased the shouting wind along and flung
My eager craft through footless halls of air.
Up, up the long delirious, burning blue
I've topped the wind-swept heights with easy grace,
Where never lark, or even eagle flew;
And, while with silent, lifting mind I've trod
The high untrespassed sanctity of space,
Put out my hand, and touched the face of God.

Lord, guard and guide.

ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals:

Dr. Alison O'Brien - for showing me that it is possible to integrate beauty, humor, and kindness with a great scientific mind, in addition to her steadfastness as my advisor

Dr. Randall Holmes - for his leadership and scientific insight with this project

Dr. John Hay - for acting as my Committee chairperson

Dr. John Newland - for EVERYTHING (enough said)

Dr. Nancy Strockbine - for her technical assistance with this project

Dr. Susan Langreth, Dr. Kathryn Holmes, and Ms. Ina Ifrim - for their individual technical assistance, instruction, and patience in teaching me transmission electron microscopy

Dr. Paul Rick - for the hunting stories that helped me get through each day

Miss Shelley Wistar and Mr. Thomas Lively - for being the "best friends" a man could wish for

Ms. Diana Fertsch, Mrs. Lydia Falk, and Mr. Jace Hougland - for the "breakfasts" and the never-ending study sessions

Lt. Col. Biever and Lt. Col. Cairney - for directing me to this strange but challenging and exciting world

Mom and Dad - for having me

My wife - for loving me

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INTRODUCTION

Significance

Acute diarrheal disease may be defined as the frequent evacuation of watery stools. Blood, mucous, and undigested food may be detected in the stools. Diarrheal disease is a worldwide problem. The disease occurs in such diverse geographical locations as the Arctic circle and the tropics. Acute diarrheal disease occurs at all ages, with the greatest incidence occurring among children less than five years of age. Fatality, as a consequence of acute diarrheal disease, is highest in lesser developed countries. Acute diarrheal diseases are a major cause of morbidity and mortality throughout the world and especially affect young children in developing countries (Gordon, 1971).

Morbidity, due to acute diarrheal diseases, is highest in children under two years of age. In 1980, the average number of episodes per child under five years of age per year was 2.2. Children under two years of age displayed the highest mortality rate: an average rate of 20/1000 population. Based on an extrapolation of these data, the estimated incidence of diarrheal diseases in 1980 was 774 million to 1 billion cases and 4.6 million deaths in children under five years of age in developing nations (excluding China) (Merson, 1982). These estimates are similar to findings from long-term field investigations on case incidence of acute diarrheal disease in India and Guatemala (Gordon, 1971).

In the last decade, at least 25 etiological agents have been implicated in acute diarrheal disease (Merson, 1982). Escherichia coli is a bacterial agent responsible for acute diarrheal disease and, thus, it has a major impact on world health (O'Brien et al., 1982a). Investigation of the molecular mechanisms of toxinogenesis in Escherichia coli is important because results obtained may offer new possibilities for developing effective vaccines. Furthermore, a greater understanding of the pathogenesis of Escherichia coli in gastrointestinal infections may be realized.

Classical Mechanisms by Which Escherichia coli Can Mediate Diarrheal Disea

Two classical mechanisms exist by which Escherichia coli can cause acute diarrheal disease in man (Dupont et al., 1971). These mechanisms are referred to as the enterotoxinogenic and enteroinvasive mechanisms. Enterotoxins may be defined as toxins which exert their primary effect on the gut and are recognized by the ability to cause fluid accumulation in ligated segments of rabbit ileum (Formal, 1961). Smith and Halls (1967) showed that a heat-stable enterotoxin (ST) could be obtained in the supernatant of aerated broth cultures of pathogenic Escherichia coli strains. Production of the heat-stable enterotoxin was shown to be determined by a transmissable plasmid (Smith and Halls, 1968). The heat-stable enterotoxin produced by certain Escherichia coli strains elicits fluid secretion by stimulation of guanylate cyclase in intestinal epithelial cells (Hughes et al., 1978; Field et al., 1978). Gyles and Barnum (1969) reported their detection of a heat-labile enterotoxin (LT) in lysates of pathogenic Escherichia coli strains. Production

of the heat-labile enterotoxin was also shown to be determined by a plasmid that could be transmitted to other strains of Escherichia coli (Gyles, 1971). The heat-labile enterotoxin produced by some Escherichia coli strains causes fluid secretion by stimulation of adenylate cyclase in intestinal epithelial cells (Evans et al., 1972). Enterotoxinogenic Escherichia coli strains do not penetrate the intestinal epithelial cells but usually adhere to the cells via host-specific colonization factors (Dupont et al., 1971). Enterotoxinogenic Escherichia coli strains are responsible for 75% of the cases of travelers' disease in adults (Lee and Kean, 1978). These enterotoxinogenic strains are also responsible for sporadic outbreaks of diarrhea in animals and in people (Dupont et al., 1971; Gyles, 1971; O'Brien et al., 1982a).

The second classical mechanism by which Escherichia coli can cause acute diarrheal disease is the enteroinvasive mechanism. This mechanism involves the bacterial invasion of colonic epithelial cells and multiplication within the cells (Dupont et al., 1971). The presence of a high-molecular-weight plasmid is closely associated with invasiveness in all enteroinvasive Escherichia coli strains (Silva et al., 1982). Enteroinvasive Escherichia coli are serologically related to shigellae and cause symptoms similar to those in shigellosis (Ogawa et al., 1968; Formal et al., 1978). Enteroinvasive Escherichia coli may be identified by their ability to produce inflammatory keroconjunctivitis in the guinea pig (Sereny, 1955). One dozen serotypes of enteroinvasive Escherichia coli have been defined and disease occurrence due to these strains is negligible in the United States (Rowe, 1977). (Refer to Figure 1).

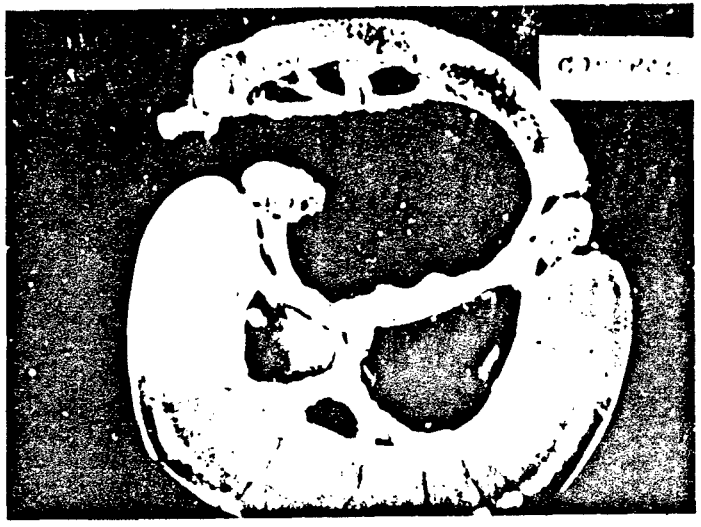


Figure 1a. Fluid accumulation in ligated segments of rabbit ileum.
(Permission for reproduction was obtained from Dr. Samuel B. Formal.)

Figure 1b. Sereny test: inflammatory kerocon-junctivitis in the guinea pig eye.

Enteropathogenic Escherichia coli Cause Acute Diarrheal Disease

Escherichia coli is capable of causing acute diarrheal disease by other means that are distinct from the classical enterotoxinogenic or enteroinvasive mechanisms (O'Brien et al., 1982a). A group of Escherichia coli strains has been isolated from young animals and children with acute diarrheal disease (Orskov et al., 1960; Rowe, 1977; Gurwith et al., 1978). These are referred to as enteropathogenic Escherichia coli or EPEC strains (Rowe, 1977). An Escherichia coli strain is so classified if it is isolated from a case of acute diarrheal disease and belongs to one of the following O serogroups: 026, 055, 0111, 0114, 0119, 0125, 0126, 0127, 0128, and 0142 (Rowe, 1977). Gurwith et al. (1978) examined several enteropathogenic Escherichia coli strains isolated in the 1940s and 1950s to see if they mediated gastroenteritis by enterotoxinogenic or enteroinvasive mechanisms. Many enteropathogenic Escherichia coli did not possess either of the two classical mechanisms known to occur in Escherichia coli (Gurwith et al., 1978). These strains did not produce detectable amounts of heat-labile enterotoxin or heat-stable enterotoxin, as ascertained by the Y-1 adrenal cell assay (Sack and Sack, 1975) or infant mouse assay (Dean et al., 1972), respectively. These EPEC strains also did not produce inflammatory keroconjunctivitis (Sereny, 1955) in the guinea pig eye (Gurwith et al., 1978). No established mechanism had been elucidated for these strains, but Levine et al. (1978) showed that two enteropathogenic Escherichia coli strains isolated from outbreaks of infantile diarrhea could produce diarrhea in adult volunteers. One interpretation of the results of this volunteer study was that the virulence of enteropathogenic Escherichia coli may

depend on factors other than the production of heat-labile or heat-stable enterotoxins or invasiveness (Levine et al., 1978).

Recent reports on Escherichia coli 015 strain RDEC-1 and Escherichia coli 0125 strain NC support this theory and shed some light on possible pathogenic mechanisms of enteropathogenic Escherichia coli strains. Cantey and Blake (1977) isclated Escherichia coli 015 strain RDEC-1 from several rabbits with diarrhea and found that when fed to young rabbits, the strain would cause diarrhea and death. Upon characterization of the RDEC-1 strain, it was not found to be enterotoxinogenic or enteroinvasive (Cantey and Blake, 1977). Electron microscopic studies performed by Takeuchi et al. (1978) indicated that RDEC-1 adhered to the small bowel mucosal epithelial cells and caused damage to the microvilli (Clausen and Christie, 1982; Rothbaum et al., 1982).

Escherichia coli Strains and Shiga-Like Toxin

Takeuchi and colleagues (1978) first proposed that destruction of the microvilli by enteropathogenic Escherichia coli strains could be due to a cytotoxin delivered to the intestinal epithelium during association of the microbe with the bowel. Formal et al. (1978) then suggested that such a cytotoxin might be related to the Shiga toxin produced by Shigella dysenteriae 1 and other Shigella species (O'Brien et al., 1977a; Keusch and Jacewicz, 1977). Shiga-like toxin seemed a viable choice as mediating diarrheal disease in the rabbit model because highly purified Shiga toxin displayed both enterotoxic and cytotoxic activities (O'Brien et al., 1980; Brown et al., 1982). O'Brien et al. (1977) tested this hypothesis and reported that Escherichia coli strain

RDEC-1 produced a cell-associated toxin that was neutralizable by anti-Shiga toxin and enterotoxinogenic for rabbit ileal loops and cytotoxic for HeLa cells. The cytotoxin was nearly identical to Shigella dysenteriae 1 toxin and was referred to as Shiga-like toxin (O'Brien et al., 1982b). Shiga-like toxin is produced in varying amounts by some strains of Escherichia coli and is defined by its ability to be neutralized by antibodies prepared against purified Shiga toxin (O'Brien et al., 1982a).

One such strain of Escherichia coli of the serotype 0157:H7 was recently reported as being a causative agent of hemorrhagic colitis in both Canada and the United States (Morb. Mortal. Wkly. Rep., 1982; Stewart et al., 1983). Escherichia coli 0157:H7 was also isolated from feces of patients with hemolytic-uremic syndrome (Karmali et al., 1983). Riley et al. (1983) tested several Escherichia coli 0157:H7 isolates for enterotoxinogenicity and enteroinvasiveness. Riley found that none of the Escherichia coli strains were enteroinvasive or enterotoxinogenic. However, Farmer et al. (1983) demonstrated the virulence of Escherichia coli 0157:H7 when he showed that infant rabbits developed diarrhea when fed Escherichia coli 0157:H7. Johnson et al. (1983) then reported that Escherichia coli 0157:H7 produced a cytotoxin other than heat-labile or heat-stable enterotoxin. O'Brien et al. (1983a) showed that the cytotoxin could be completely neutralized by antitoxin to purified Shiga toxin. O'Brien et al. (1983b) then reported that the level of Shiga-like toxin produced by three Escherichia coli 0157:H7 strains was equivalent to that of Shigella dysenteriae 1 (Table 1). For example, the level of toxin made by Escherichia coli 0157:H7 strain 933 was approximately 10^5 50 percent cytotoxic doses per milliliter of culture

Table 1.

CD_{50} = cytotoxic dose required to kill 50% of cells in microtitre assay. (Expressed as per ml culture supernatant or as per mg protein in bacterial lysates.) For lysate studies organisms were cultured 48 h in 500 ml of iron-depleted modified syncase broth, harvested by centrifugation, washed twice in saline (0.85%), resuspended in 3 ml saline, and cells were disrupted by sonic lysis. Lysates were clarified by centrifugation and protein concentrations were estimated spectrophotometrically.

Neutralization of cytotoxicity was defined by antiserum dilutions of $\leq$ 1/1600 completely protecting HeLa and Vero cells from 10 CD_{50} of toxin in culture supernatants and cell lysates. A 1/1000 dilution of prebleed serum did not neutralize the toxicity of these samples. Controls included purified Shiga 60R toxin and E. coli H30 toxin. The titre of each toxin was 10^9 CD_{50}/mg toxin when tested on either HeLa cells or on Vero cells. Both toxins were completely neutralized by antitoxin but not by prebleed serum (O'Brien et al., 1983c).

Table 1. CYTOTOXICITY OF E. COLI 0157:H7 BACTERIAL LYSATES AND CULTURE FILTRATES FOR VERO AND HeLa CELLS

Strain*	Cytotoxicity for HeLa cells		Cytotoxicity for Vero cells		Cytotoxicity neutralized by rabbit antiserum to purified Shiga toxin
	CD_{50}/ml	CD_{50}/mg	CD_{50}/ml	CD_{50}/mg	
A	10^5	$2x10^7$	10^6	$3x10^5$	Yes
B	10^5	$8x10^4$	10^5	$2x10^5$	Yes
C	10^5	$8x10^5$	10^6	$2x10^6$	Yes

*Strain A = EDL 931 (human); strain B = EDL 932 (human); strain C = EDL 933 (hamburger). (CD_{50}/ml refers to culture filtrates and CD_{50}/mg refers to bacterial lysates.)

supernatant and 10^6 50 percent cytotoxic doses per milligram of protein in lysates of bacteria. In a further study, O'Brien et al. (1983c) then purified Escherichia coli 0157:H7 strain 933 toxin to homogeneity. The pure toxin from Escherichia coli 0157:H7 strain 933 possessed the identical multiple B : one A subunit structure as seen with Shigella dysenteriae 1 strain 60R (Figure 2) (Olsnes et al., 1981). Furthermore, the pure toxin from Escherichia coli 0157:H7 strain 933 had the same biological activities as purified toxin from Shigella dysenteriae 1 strain 60R (O'Brien et al., 1983c).

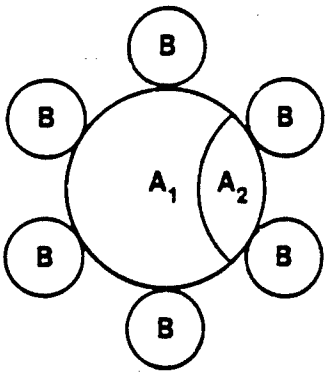
Escherichia coli Strains and Shiga-Like Toxin Converting Phages

Scotland et al. (1983) reported that the genes for Vero cell cytotoxin production from Escherichia coli 026 strain H-19 were carried on a bacteriophage. Escherichia coli 026 strain H-19 does not produce heat-labile or heat-stable enterotoxin and is noninvasive. This Escherichia coli strain was isolated from an outbreak of infantile diarrhea in Great Britain more than 15 years ago (Smith and Linggood, 1971). Escherichia coli 026 strain H-19 produces large amounts of Shiga-like toxin (Konawalchuk et al., 1977; O'Brien et al., 1983a). Shiga-like toxin, which is cytotoxic for HeLa cells, is also cytotoxic for Vero cells and was originally called Vero cell cytotoxin (Scotland et al., 1983; Konawalchuk et al., 1977; O'Brien et al., 1983a). The toxin-converting phage was released spontaneously from a plasmid-negative Escherichia coli K-12 strain that had become toxinogenic after cocultivation with Escherichia coli 026 strain H-19. Attempts to isolate the bacteriophage from Escherichia coli 026 strain H-19 were unsuccessful

Figure 2.

Model for Structure of Shiga Toxin

MW = ~65,000



$$A = 30{,}000 \xrightarrow[2ME]{Tryp} \begin{matrix} A_1\ (27{,}000) + A_2\ (3{,}000) \\ A_1 \text{ inhibits protein synthesis} \end{matrix}$$

B = ~7,000, binds to cell receptor

Taken from data of Olsnes, Reisbig & Eiklid, J. Biol. Chem. 1981, 256:8732-8738 and O'Brien & LaVeck, Infect. Immun. 1983, 40:675-683.

even after mitomycin induction (Scotland et al., 1983). However, it was confirmed that Escherichia coli strain K-12 when treated with purified Vero cell cytotoxin converting phage became lysogenic and produced a cytotoxin which acted on Vero cells (Scotland et al., 1983). Electron microscopic studies of the cytotoxin converting phage showed the phage particles had elongated hexagonal heads and non-contractile flexible tails. The estimated molecular weight of the bacteriophage was approximately 30×10^6 (Scotland et al., 1983).

Smith et al. (1983) then demonstrated that two different cytotoxin converting phages were released from Escherichia coli 026 strain H-19. The probable reason for Escherichia coli 026 strain H-19 previously being recorded as non-lysogenic was that broth cultures of strain H-19 contained fewer than 10 ml^{-1} phage particles. Cytotoxin-converting phages were also isolated from Escherichia coli strains H28 and H12 (Smith et al., 1983). O'Brien et al. (1984) also demonstrated that a cytotoxin-converting phage could be induced from Escherichia coli 026 strain H-19 upon ultraviolet-light induction of the bacterial strain. O'Brien and her colleagues (1984) then proceeded to characterize the phage which had been isolated from strain H-19.

Specific Aims

When this project was initiated (July 1983), it had been shown that some Escherichia coli strains associated with disease in man produce high levels of Shiga-like toxin but are not enteroinvasive nor are they able to make heat-labile or heat-stable toxins. It had also been established that one such strain, Escherichia coli H-19, harbored Shiga-

like toxin-converting phages. At that time, neither of the H-19 phages had been fully characterized. Moreover, no phage had been isolated from Escherichia coli 0157:H7. Therefore, the goal of these studies was to isolate a Shiga-like toxin-converting phage from Escherichia coli 0157:H7 strain 933 and analyze the bacteriophage at a molecular level. With this goal, the following specific aims were drawn:

I. Isolate a Shiga-like toxin-converting phage from Escherichia coli 0157:H7 strain 933.

II. Characterize the morphology, structural polypeptides, and DNA restriction fragment pattern of the strain 933 phage and then compare these criteria to one of the previously isolated strain H-19 phages.

III. Isolate and purify the strain 933 phage DNA.

IV. Prepare a detailed restriction enzyme map of phage 933 DNA and assist Dr. John Newland in cloning and subcloning the structural genes responsible for Shiga-like toxin production.

V. Construct a detailed map of restriction endonuclease cleavage sites within the subcloned DNA of the phage.

VI. Initiate molecular epidemiologic studies in which strains of Escherichia coli and other bacteria from clinical and natural sources are probed with internal sequences of the Shiga-like toxin structural genes.

METHODS AND MATERIALS

Bacterial Strains

Escherichia coli 0157:H7 strain 933 (obtained from Dr. George Morris at the Centers for Disease Control) had been isolated from a food-borne outbreak of hemorrhagic colitis in humans from Michigan. Escherichia coli 026 strain H-19 (Smith and Linggood, 1971) was isolated from an outbreak of infantile diarrhea in Great Britain. These Escherichia coli strains do not produce heat-labile or heat-stable enterotoxins and are noninvasive. However, these strains do produce significant amounts of Shiga-like toxin. Strain 933 contains two Shiga-like toxin converting phages, designated 933J and 933W, which differ from each other in immunity specificity and resistance to thermal inactivation. Strain H-19 also harbors two distinct toxin-converting phages: H-19A/J (independent isolates of the same phage) and H-19B. These two phages differ from each other only in immunity specificity (O'Brien et al., 1984). Escherichia coli strains 395-1 and c600 are substrains of Escherichia coli K-12 and were used both in plaquing experiments and in constructing lysogens with the above-mentioned phages [i.e., c600(H-19J) or 395-1(H-19J)]. Escherichia coli strain DS410 and Escherichia coli strain HB101 were used in minicell analysis and cloning experiments, respectively.

Culture Media and Diluents

Bacteria were grown in Penassay broth (Difco, Detroit, MI), Chelex-treated syncase medium (Difco, Detroit, MI), LB broth (Miller, 1972), or LB broth prepared with half the original amount of NaCl and supplemented with 10mM $CaCl_2$ and 0.001 percent thiamine (designated modified LB broth). The selection of a particular medium was dictated by the nature of the experiment. Samples of bacterial lysates or culture filtrates were diluted for cytotoxicity assays in HeLa cell tissue culture medium that consisted of Minimal Eagle's Essential Medium (MA Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS, Flow Laboratories), 0.1% L-glutamine (FBS, Flow Laboratories), 50 µg/ml gentamicin (Quality Biological Inc., Gaithersburg, MD) and 100,000 µg/ml penicillin G (FBS, Flow Laboratories). Large-scale bacteriophage harvests were stored in modified lambda phage diluent (Maniatis et al., 1982) that contained half the original amount of NaCl. Minicells were diluted in BSG media (Frazer and Curtis, 1975), M9 media (Frazer and Curtis, 1975), and M9 media supplemented with methionine assay media (Difco, Detroit, MI). Purified phage DNA preparations were stored in TE buffer (Miller, 1972).

Ultraviolet Light Bacteriophage Induction

Bacterial cultures (5 ml) were grown in modified LB broth to an optical density of 0.5 at a 600 nm wavelength. The bacteria were collected by centrifugation and resuspended in 5 ml of 10 mM $CaCl_2$. The $CaCl_2$-suspended cells were then irradiated with 40 J/m^2 of ultraviolet

light in 100 mm glass petri dishes. Ten-fold serial dilutions of the irradiated bacteria were prepared in modified LB broth and then incubated for 5 hours at 37°C in foil-covered tubes. Two drops of chloroform were added to each tube, and cellular debris was removed by low-speed centrifugation. The lysates were then sterilized by membrane filtration (0.45 microns, Nalgene, Rochester, NY) and stored at 4°C.

Plaque Assays, Plaque Purification, and Lysogen Construction

Escherichia coli K-12 substrain 395-1 (Sansonetti et al., 1983) was used as the indicator strain for all plaque assays. Indicator cell cultures (0.1 ml) were grown to an optical density of 0.5 at a 600 nm wavelength, and these cells were used to adsorb ten-fold serial dilutions of the phage (0.1 ml) in modified LB broth for 20 minutes at 37°C. Aliquots (100 µl) of the adsorption mixtures were added to 3 ml of 0.7% modified LB overlay agar. This mixture was then poured into petri dishes that contained 1.5% modified LB bottom agar. The plates were then incubated at 37°C. Plaques were counted the following day.

An individual plaque was stabbed with a sterile, platinum wire and inoculated into 1 ml of modified LB broth. The inoculum was then plated onto indicator cells as previously discussed. This process was repeated four consecutive times.

Lysogens were then constructed with plaque-purified phages. A sterile, platinum wire was used to pick the center of an individual plaque isolated on Escherichia coli K-12 substrain 395-1 or c600. This inoculum was then streaked for isolation over the surface of hard, modified LB medium, and the plates were incubated overnight at 37°C.

Resultant colonies were then screened for lysogenicity by their ability to release phage upon irradiation with ultraviolet light (as previously discussed).

Large-Scale Harvest of Phage Lysates

Bacteriophages were plated onto indicator cells as previously described. Each plate that contained between 10^3 and 10^4 plaque forming units (PFU) was soaked with 4 ml of a modified lambda-phage diluent and stored overnight at 4°C. The aqueous phase was eluted from each plate and the eluates were pooled to form a large-scale phage preparation. This preparation was then sterilized by membrane filtration and stored at 4°C.

HeLa Cell Assay for Determination of Toxin-Converting Phages

Escherichia coli K-12 substrain 395-1 was used as the host strain in phage conversion experiments. Large plaques that developed after exposure of substrain 395-1 to phage lysates were excised with Pasteur pipets and inoculated into 1 ml samples of Penassay broth. After overnight incubation at 37°C, the broth cultures were centrifuged and culture supernatants were diluted 1:250 in HeLa cell tissue culture medium. Samples (100 µl) were then tested on HeLa cells for cytotoxicity.

Assay for Production of Low Levels of Shiga-Like Toxin

Lysogens were streaked for isolation onto LB medium, and incubated overnight at 37°C. Isolated colonies were then picked, inoculated into 50 ml of Penassay broth, and cultures were grown overnight in a shaking waterbath at 37°C. A sample (25 µl) of this overnight culture was then inoculated into 500 ml of Chelex-treated syncase media and grown for 48 hours at 37°C. After centrifugation at 5,000xg for 10 minutes, an aliquot of the supernatant was frozen and saved for use in HeLa cell assays. The pellet was then washed twice with saline and resuspended in phosphage-buffered (Difco, Detroit, MI) saline (3 ml). This suspension was sonicated (Bronson, Danbury, CT) for three minutes, and the sonic lysate was frozen at -20°C. HeLa cell assays (previously discussed) were then performed on both the supernatants and the sonic lysates (O'Brien, 1983).

Transmission Electron Microscopy of Uranyl-Acetate Stained Phage

High-titred phage stocks (approximately 10^{10} PFU/ml) were prepared as discussed earlier. Next, the phages were purified by CsCl step-gradient centrifugation (Maniatis et al., 1982). Purified phage were resuspended in 100 µl of modified lambda-phage diluent. A 10 µl sample of the phage suspension was placed onto a carbon-coated Formvar grid (Hayat, 1972) for 1 minute and then adsorbed with 3 MM paper (Whatman, England). TE buffer (10 µl) (Maniatis et al., 1982) was then placed onto the grid and adsorbed after 30 seconds, and the phage was stained with 10 µl of 0.5% uranyl-acetate (Sigma, St. Louis, MO) for 1

minute. Carbon-coated Formvar grids that contained stained phage specimens were viewed at a magnification of 100,000 x with a Jeol 100-CX transmission electron microscope (Jeol, Switzerland).

Analysis of Phage Structural Polypeptides

Phage preparations were plaque purified and isolated by CsCl gradient ultracentrifugation as discussed earlier. Samples of purified phages were treated with SDS-breaking buffer solution (Maniatis et al., 1982) and boiled for 10 minutes at 100°C. Aliquots of this suspension, in addition to five molecular weight standards, were then subjected to electrophoresis in a 12% sodium dodecyl sulfate-polyacrylamide gel (Miller, 1972). Gels were stained with Coomassie blue (O'Brien et al., 1983c).

Purification and Concentration of Phage DNA

CsCl gradient-isolated phages were centrifuged and resuspended in TE buffer. Phage samples were mixed with an equal volume of phenol in a polypropylene tube, and the contents were mixed until an emulsion formed. Next, the samples were centrifuged for 5 minutes in a table-top microfuge (Damon/IEC, Needham, MA) at room temperature, and the upper aqueous phage of each sample was transferred to a fresh polypropylene tube. After a second phenol extraction, two phenol-chloroform extractions and two chloroform extractions were performed in similar fashion. Phage DNA was recovered by precipitation with 100% ethanol (2x volume at 4°C) and electrophoresed on a 0.7% agarose gel (BRL, Gaithersburg, MD).

The gel was stained with ethidium bromide (Sigma, St. Louis, MO) and examined under ultraviolet light (Ultra-Violet Products, San Gabriel, CA) to visualize the DNA (Maniatis et al., 1982).

Assessment of Restriction Enzyme Nuclease Digest Patterns from Phage DNA

Phage DNA was isolated and purified as previously discussed. A 20 µl reaction mixture consisted of sterile, distilled H_2O (13 µl), the appropriate enzyme buffer (2 µl), phage DNA (4 µl), and restriction enzyme nuclease (1 µl). The reaction was allowed to proceed for 1 hour in a 37°C water bath. Enzymes used in analyzing the restriction enzyme nuclease digest patterns of phage DNA were Eco RI, Hind III, and Bam HI (BRL, Gaithersburg, MD). The reactions were determined by placing them in an ice bath, and the restriction endonuclease-digested DNA was electrophoresed in a 0.7% agarose gel and stained with ethidium bromide. The sizes of the fragments were determined by their electrophoretic mobilities relative to Hind III reference fragments of DNA from phage lambda (IBI, New Haven, CT). The size of the intact DNA from each of the bacteriophages was estimated on the basis of the sum of the individual restriction fragments.

Cloning and Subcloning of Phage Structural Genes

Cloning of the gene(s) responsible for high level production of Escherichia coli Shiga-like toxin was performed under P4 + EK1 conditions (as specified by the NIH Recombinant DNA Advisory Committee). Further subcloning of the bacteriophage structural gene(s) was carried

out under P3 laboratory conditions (NIH Recombinant DNA Advisory Committee). Cloning was performed in the plasmid vector pBR328 (Boehringer Mannheim, Indianopolis, IN) which contains three antibiotic-resistance markers as shown in Figure 3. Directional cloning and insertional inactivation were the primary strategies used in the cloning experiments.

Plasmid DNA was isolated in the following manner. Cultures (5 ml) of plasmid-containing bacteria were grown overnight in modified LB broth supplemented with 10 µg/ml tetracycline (Sigma, St. Louis, MO) at 37°C. Next, cultures were subjected to centrifugation, and the pellets were resuspended in 100 µl of lysozyme solution (Davis, 1980) and chilled on ice for 15 minutes. This solution was treated with 10 N NaOH and SDS, chilled on ice for 5 minutes, and precipitated with 3 M Na acetate (pH 4.8). Plasmid DNA was precipitated with 100% ethanol at 4°C, and the DNA was purified and concentrated as previously discussed. Phage DNA fragments to be subcloned were electroeluted from 0.7% agarose gels as detailed in the instruction book from an electroelution kit developed by International Biotechnologies, Inc. (IBI, New Haven, CT).

Linearized plasmid DNA (200 ng) was mixed with a three-fold molar excess of the fragment to be subcloned. This mixture of DNA was precipitated with 100% ethanol and washed with 70% ethanol. Next, the precipitate was dissolved in a solution of 8 µl of TE buffer (pH 8.0), 1 µl of 10x ligation buffer (Maniatis et al., 1982) and 10 units of T4 ligase (IBI, New Haven, CT), and incubated overnight at 12°C. Successful ligation was checked by agarose gel electrophoresis (Maniatis et al., 1982).

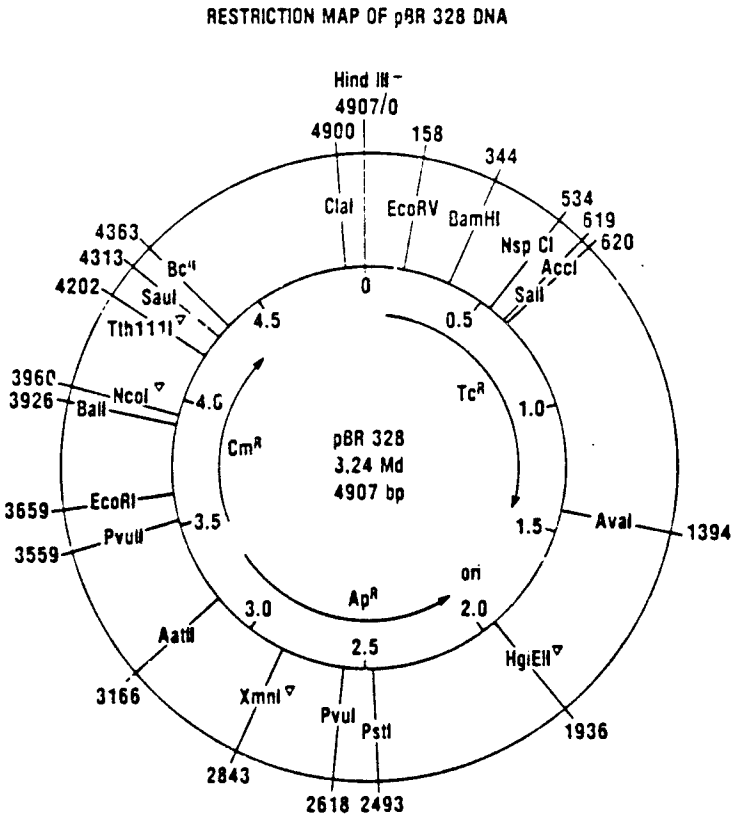


Figure 3. Restriction map of the plasmid pBR328 (Boehringer Mannheim, 1983).

Plasmid DNA was introduced into Escherichia coli HB101 by transformation. Escherichia coli HB101 was cultured and rendered competent for transformation as follows. LB broth (40 ml) was inoculated with 1 ml of an overnight HB101 culture and grown to an optical density of 0.5 (600 nm wavelength). The culture was then placed on ice for 1 hour, and the cells were collected by centrifugation at 4000xg for 5 minutes at 4°C. Next, the cell pellet was resuspended in 20 ml of 50 mM $CaCl_2$ and 10 mM Tris-Cl (pH 8.0) and chilled on ice for 30 minutes. The treated bacteria were harvested by centrifugation and resuspended in 5 ml of 50 mM $CaCl_2$ solution. The protocol by which these competent bacteria was transformed with DNA was as follows. DNA suspended in TE buffer (100 µl) was added to 100 µl of the bacteria suspended in the $CaCl_2$ solution and the mixture was then heated at 42°C for 2 minutes. This suspension was added to LB broth (1 ml) and incubated for 1 hour at 37°C to allow the bacteria to express the plasmid-encoded antibiotic resistance genes. Transformation mixtures were then added to LB overlay agar (2 ml), the overlay poured onto LB bottom agar, and the plates incubated in an inverted position at 37°C overnight. Finally, transformant colonies were identified by plasmid-encoded resistance to antibiotics and subsequently tested for cytotoxicity as described earlier.

Analysis of Plasmid-Encoded Proteins

Plasmids that contained selected subclones were transformed into the minicell-producing strain Escherichia coli DS410 by the same procedure used to transform Escherichia coli HB101. An isolated colony from each type of transformant was inoculated into 200 ml of LB broth,

and the cultures were incubated overnight at 37°C with shaking. The cultures were then chilled on ice, the bacteria collected by centrifugation, washed in 10 ml of BSG (Frazer and Curtis, 1975), and resuspended in 2 ml of BSG. Minicells were then separated from intact bacteria by sucrose step-gradients (Frazer and Curtis, 1975), suspended in 20 ml of BSG, and purified by continuous sucrose gradients (Frazer and Curtis, 1975). The purified minicells were then diluted 1 to 2 in MMM (Frazer and Curtis, 1975), pelleted by centrifugation, washed two times in MMM, diluted with MMM to an optical density of 1.0 (600 nm wavelength), and incubated with shaking for 45 minutes at 37°C. Next, [^{35}S]-methionine (Frazer and Curtis, 1975) was added to the cultures and they were then incubated 1 hour at 37°C. The radiolabeled minicells were then pelleted by centrifugation. The pellets were resuspended in 100 µl of cracking buffer plus 25 µl of sample buffer (Frazer and Curtis, 1975) and heated at 100°C for 5 minutes. Samples were subjected to electrophoresis on a sodium dodecyl sulfate, 12% polyacrylamide gel. The gel was then dried and examined by fluorography (Maniatis *et al.*, 1982).

Alpha-^{32}P Labeling of Subcloned Phage DNA by Nick Translation

Subcloned phage DNA was radiolabeled according to the instructions with a nick-translation kit purchased from Amersham Corporation (Amersham Corp., Arlington Heights, IL). A 50 µl reaction mixture that contained 27 µl of sterile distilled H_2O, 3 µl of DNA (1 µg/ml), 5 µl of nick-translation buffer, 10 µl of ^{32}P-labeled dCTP, and 5 µl of an *Escherichia coli* DNA polymerase I - DNase mixture was incubated for 2 hours at 15°C. The reaction was terminated by addition of 5 µl of "stop

buffer." Gel filtration was used to separate free label from labeled DNA. Siliconized glassware was used to construct a column containing 100-200 mesh P60 beads (Bio Rad, Richmond, CA) suspended in TE buffer. The column was equilibrated with TE buffer and then the reaction mixture was loaded into the P60 column. The column was washed with TE buffer, and 0.5 ml effluent samples were collected in polypropylene tubes. Radioactivity of the column fractions was monitored with a Geiger counter (Ludlum). The ^{32}P-labeled DNA was located in the third and fourth fractions (200 µl). Radio-labeled DNA was stored at 4°C.

Preparation of Bacterial Chromosomal DNA for Southern Blots

An isolated colony from each bacterial strain was inoculated into LB broth (30 ml) and grown overnight at 37°C. These bacteria were then harvested by centrifugation at 5000Xg for 10 minutes, resuspended in 5 ml of STE (Davis et al., 1980) modified with 15% sucrose and supplemented with 1 µg/ml lysozyme, and the suspension chilled on ice for 30 minutes. Next, 500 µl of a 10% sodium dodecyl-sulfate solution and 100 µl of a 2 mg/ml (in H_2O) stock solution of proteinase K (Sigma, St. Louis, MO) were added to the lysozyme-treated bacteria. The mixture was then heated at 56°C for 15 minutes. Each bacterial digest was then mixed with an equal volume of chloroform:phenol (50/50), shaken gently for 15 minutes, subjected to centrifugation, and the upper aqueous phase of the extract removed. Chromosomal DNA in this aqueous phase was precipitated with two volumes of 100% ethanol (4°C) and collected on a glass micropipet. The DNA was dissolved in 1 ml of TE buffer, containing RNase (Sigma, St. Louis, MO) to a final concentration of 50

µg/ml, incubated for 1 hour at 37°C, and stored at 4°C. The chromosomal DNA was then digested with Eco RI and subjected to electrophoresis in a 0.7% agarose gel, stained with ethidium bromide (Silhavey et al., 1984).

Southern Transfer of Chromosomal DNA from Agarose Gels to Nitrocellulose Paper

A gel that contained electrophoretically-separated chromosomal DNA fragments was placed in a glass baking dish. Areas of the gel that did not contain DNA were trimmed away with a razor blade. The DNA was denatured by soaking the gel in several volumes of 1.5 M NaCl and 0.5 M NaOH (Southern, 1975) for 1 hour at room temperature with constant shaking. The gel was then neutralized by soaking it in several volumes of 1 M Tris-Cl (pH 8.0) and 1.5 M NaCl (Southern, 1975) for 1 hour at room temperature with constant shaking. A plexiglass support wrapped with a piece of 3 MM paper (Whatman, England) was placed in a large baking dish filled with 20X SSPE (salt-sodium phosphate-EDTA) (Maniatis, 1982) and air bubbles trapped under the 3 MM paper were pressed out with a glass rod. Next, the gel was inverted and placed on top of the damp 3 MM paper (care was taken to avoid air bubbles between the 3 MM paper and the gel). A nitrocellulose filter moistened with 20X SSPE was placed on top of the gel and air bubbles were again pressed out with a glass rod. Two pieces of 3 MM paper were placed on top of the nitrocellulose along with a 5-8 cm high stack of paper towels. A glass plate was placed on the top of this stack, and the plate was weighed down with a 500 gram weight. After allowing the transfer of DNA to proceed for 24 hours, the positions were marked with a pen. The filter was then soaked in 5X SSPE

for 10 minutes, dried at room temperature, and baked for 2 hours at 80°C under vacuum (Maniatis et al., 1982).

Hybridization to DNA on a Solid Support

The filter to which chromosomal DNA was bound was placed in a heat-sealable plastic bag with 8 ml of hybridization mix (Davis et al., 1980) that was supplemented with 100 µg/ml of denatured salmon sperm DNA (Sigma, St. Louis, MO). The bag was heat-sealed and incubated at 65°C for 1 hour. Next, the nick-translated ^{32}P-labeled DNA probe was denatured by incubation of an aliquot (5×10^6 dpm) with 100 µl of 1 N NaOH for 10 minutes at room temperature and the base neutralized by addition of 100 µl of 1.8 M Tris-HCl and 0.2 M Tris base (Davis et al., 1980). The denatured probe was then added to the bag, the bag resealed, and hybridization reactions allowed to proceed for 24 hours at 65°C. Stringency conditions were adjusted in independent experiments by using the following equation: $T_m = 16.6 \text{ Log}(Na^+) + 0.41(\%G + C) + 81.5$ and by assuming for every 1% lowering of homology, the T_m is lowered by 1.5°C (Davis et al., 1980). The filter was then removed from the bag and washed several times with 2X SSPE and 0.2% SDS at 45°C with agitation. Finally, the filter was dried and covered in plastic wrap. Hybridization reactions were visualized by autoradiography with Kodak X-Omat film (Kodak, Rochester, NY).

RESULTS

Phage Induction and Phage Conversion

The method used for bacteriophage induction yielded phages from both Escherichia coli strain 933 and H-19. Lysates of strain H-19 consistently contained more infectious phage (10^9 PFU/ml) than lysates of strain 933 (10^5 PFU/ml). Figure 4 shows the morphology of specific plaques that formed on the indicator strain Escherichia coli K-12: 395-1 after it was exposed to a lysate of strain 933. Plaques isolated from 395-1 after exposure to each lysate exhibited consistent morphological characteristics in independent experiments. The plaques were between 3 and 4 millimeters in diameter, circular, and turbid in appearance.

To determine whether the phages released from Escherichia coli strains 933 and H-19 after ultraviolet light induction were toxin-converting phages, diluted culture supernatants derived from single plaques (grown in LB broth) were tested on HeLa cells for cytotoxicity. For this purpose, 50 cultures were tested from each strain independently. Undiluted supernatants from the nonlysogenized strain Escherichia coli 395-1 (not cytotoxic) were used as the negative HeLa cell assay controls, and 1:250 dilutions of supernatants from Escherichia coli 933 or Escherichia coli H-19 (cytotoxic) were used as positive HeLa cell assay controls. Each of the 50 cultures inoculated with material from single plaques of phage 933 or H-19 (isolated from lawns of Escherichia coli 395-1) contained detectable toxin. One phage isolated from

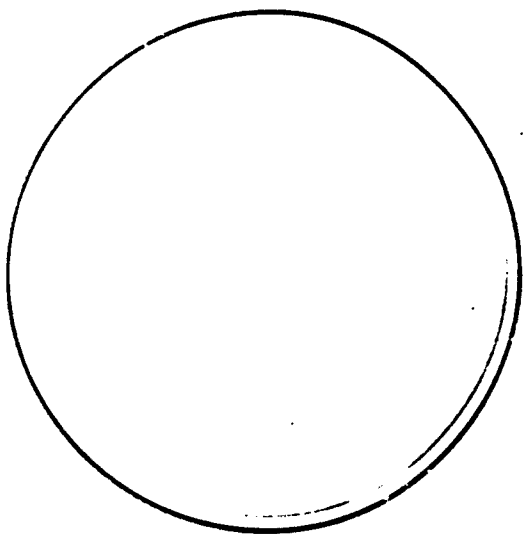


Figure 4. Morphology of specific plaques that formed on indicator strain (Escherichia coli K-12: 395-1), after exposure to a lysate of strain 933.

Escherichia coli 933, designated 933J, and one phage isolated from Escherichia coli H-19, designated H-19J, were subjected to plaque purification, and lysogens of Escherichia coli 395-1 and Escherichia coli C600 were constructed with each of the plaque-purified phages. After ultraviolet irradiation of both 395-1(833J) and 395-1(H-19J), the phage titres were the same (10^7 PFU/ml). Furthermore, each of these lysogens produced equivalent titres of Shiga-like toxin (Table 2), as compared to the original Escherichia coli 933 and H-19 strains (10^6 CD_{50} per milligram of protein in cell lysates and 10^5 CD_{50} per milliliter in supernatants when grown in deferrated glucose-syncase medium). However, under similar conditions, control cultures of Escherichia coli 395-1 and Escherichia coli C600 made only trace levels of Shiga-like toxin [$\leq 10^2$ CD_{50} per milligram of protein in cell lysates and no detectable toxin in culture filtrates (O'Brien et al., 1984)]. Thus, the phage isolates 933J and H-19J both converted Escherichia coli 395-1 and Escherichia coli C600 to produce high titres of Shiga-like toxin.

Morphology, Structural Polypeptide Content, and DNA Restriction Fragment Pattern of Phage 933J and Phage H-19J

The morphology, structural polypeptide content, and DNA restriction fragment patterns were compared for the two toxin-converting phages (933J and H-19J). High-titred phage stocks (10^{10} PFU/ml) were purified by centrifugation through CsCl step-gradients, and the purified phages were placed on Formvar carbon-coated grids and stained with 0.5% uranyl acetate. Individual electron micrographs (Figures 5 and 6) of both phage 933J and H-19J demonstrate that both phages have a bullet-shaped

Table 2. Titres of Shiga-like toxins produced by lysogen 395-1(933J) and 395-1(H-19J)

Strain	CD_{50}/mg protein(cell lysates)	CD_{50}/ml(supernatants)
Escherichia coli 933J	10^6	10^5
Escherichia coli H-19	10^6	10^5
Escherichia coli K-12:395-1	$\leq 10^2$	*
Escherichia coli 395-1(933J)	10^6	10^5
Escherichia coli 395-1(H-19J)	10^6	10^5

* = no detectable toxin in culture filtrates

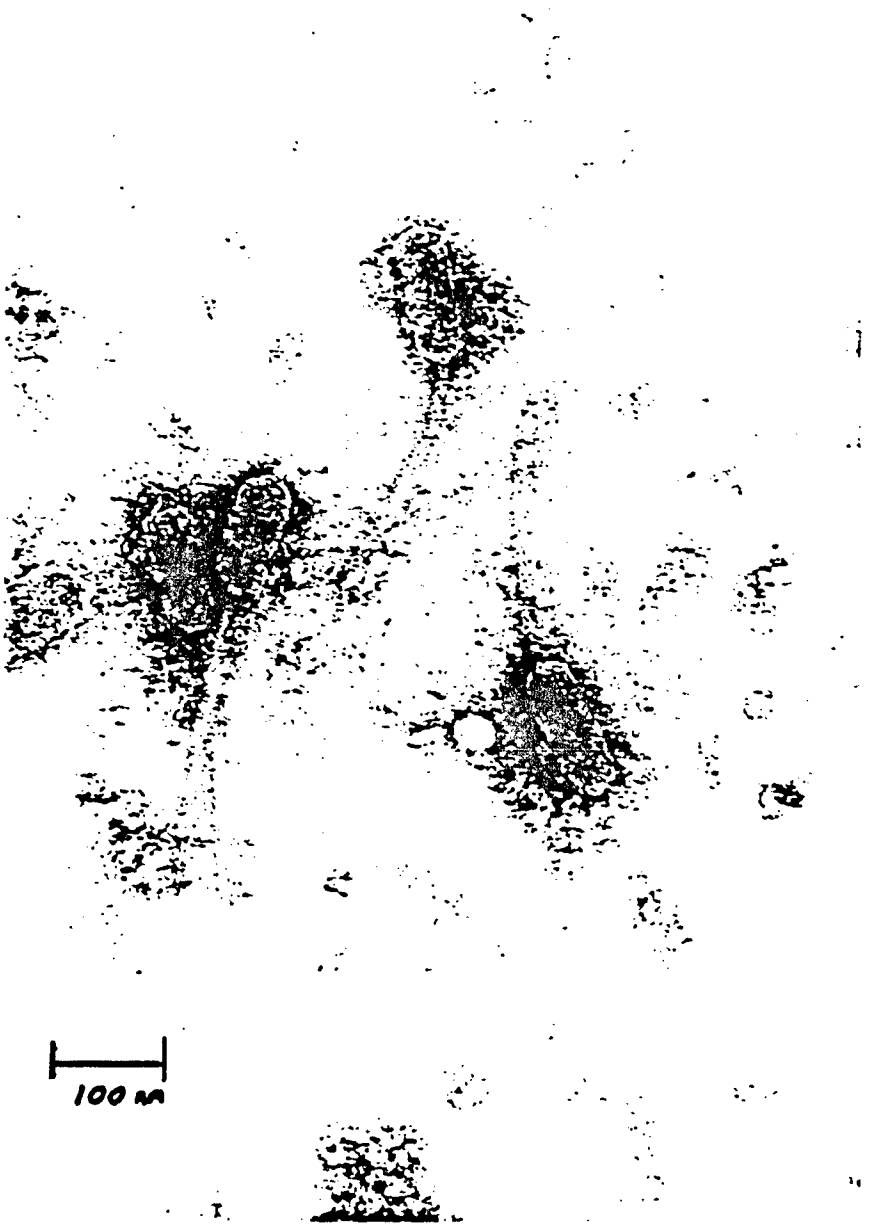


Figure 5. Electron micrograph of plaque-purified 933J phage negatively stained with uranyl acetate (pH 4.5) (O'Brien et al., 1984).

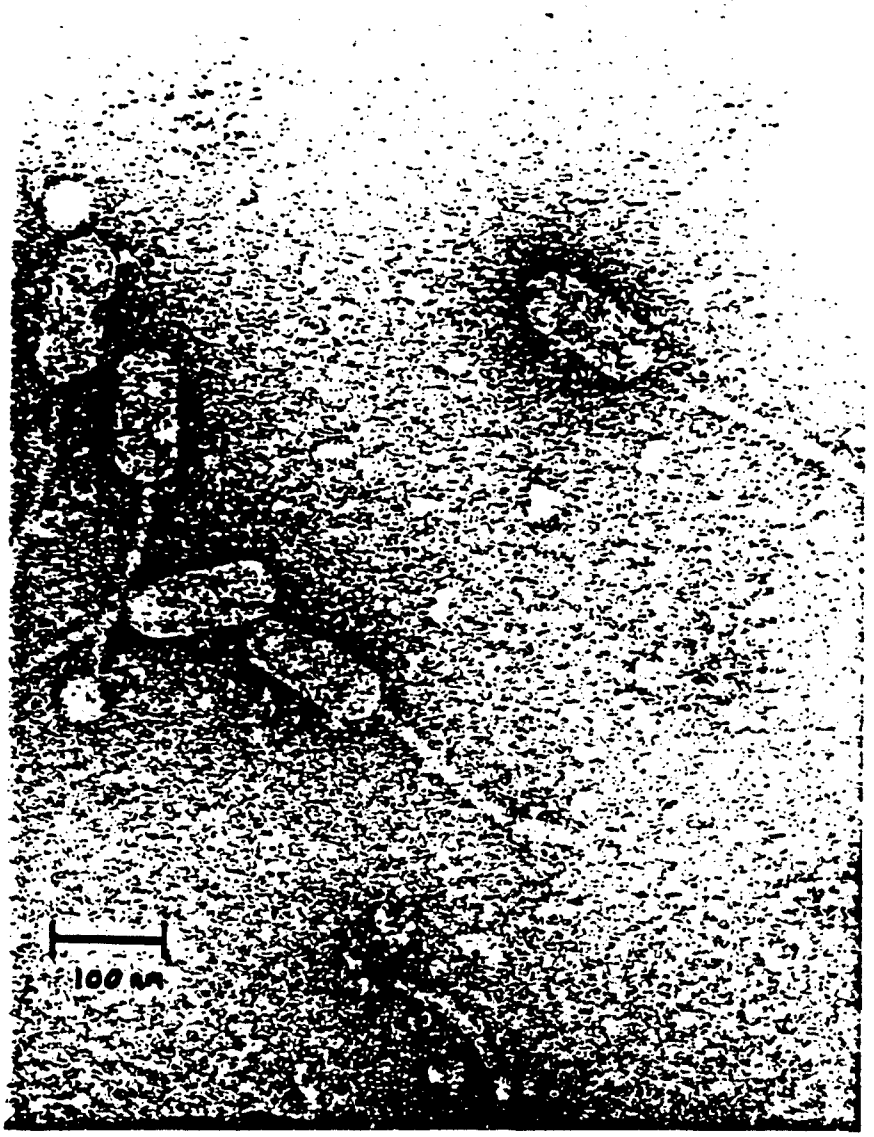


Figure 6. Electron micrograph of plaque-purified H-19J phage negatively stained with uranyl acetate (pH 4.5) (O'Brien et al., 1984).

head and a long tail. At 100,000X magnification, the dimensions of these toxin-converting phages were 120 nanometers (nm) in length for the phage head and 210 nm for the phage tail. As shown in Figure 7, the morphologies of the uranyl acetate-stained 933J and H-19J phages were indistinguishable by electron microscopy. The polypeptides of the two phages were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The results of this experiment are presented in Figure 8. Two structural polypeptides from each phage co-migrated with a 66.2 kilodalton (kD) (bovine serum albumin) and 45 kD (ovalbumin) molecular weight standards. Phage H-19J (lane A) and phage 933J (lane B) possess structural polypeptides with similar electrophoretic mobilities. DNA from plaque-purified, CsCl gradient-isolated phages 933J and H-19J were digested with restriction endonucleases Eco RI, Hind III, Bam HI, and analyzed by agarose gel electrophoresis. As shown in Figure 9 (not all bands visible), the Eco RI digest yielded 12 bands, Bam HI yielded 8 bands, and Hind III yielded 7 bands. No differences were detected between the mobilities of these bands from both toxin-converting phages. The sizes of the individual restriction fragments (Table 3) were determined by their relative electrophoretic mobilities when compared to Hind III reference fragments of DNA from phage lambda. The size of the intact DNA from each of the phages was estimated as 50-55 kilobase pairs, on the basis of the sum of the sizes of the individual restriction fragments. Moreover, ^{32}P-labeled H-19J phage DNA was hybridized in Southern blots with all restriction fragments of Eco RI digested phage 933J DNA (Table 5). Hence, these comparative studies of morphology, structural polypeptides, and DNA indicate that the Shiga-like toxin-converting phages 933J and H-19J are the same or are closely related.

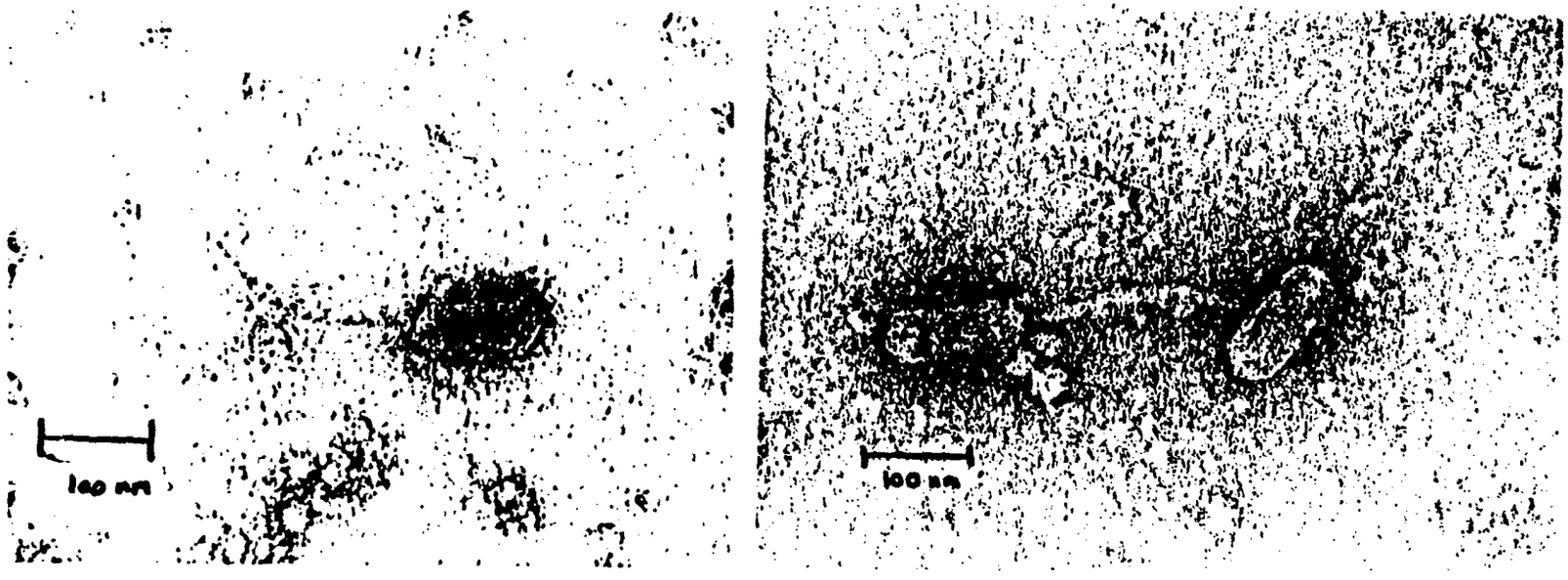


Figure 7. Electron micrograph comparison of plaque-purified 933J and H-19J phages negatively stained with uranyl acetate (pH 4.5) (O'Brien et al., 1984).

Figure 8.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of polypeptides from plaque-purified, CsCl gradient-isolated converting phages H-19J (lane A) and 933J (lane B). Lane C contains a mixture of molecular weight standards (phosphorylase B, 92.5 kD; bovine serum albumin, 66.2 kD; ovalbumin, 45 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD). Samples of phage were treated with SDS-containing buffer, subjected to electrophoresis in a 12 percent gel, and stained with Coomassie blue.

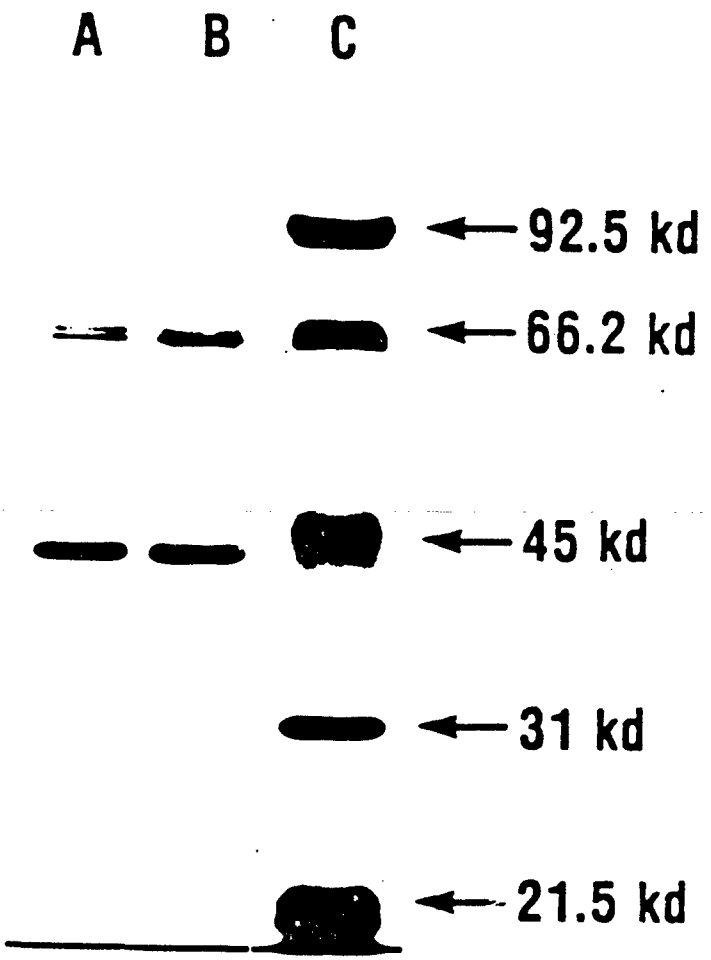


Figure 8. SDS-PAGE of polypeptides from phage H-19J and phage 933J (O'Brien et al., 1984).

Figure 9.

Agarose gel electrophoresis and ethidium bromide staining of restriction endonuclease-digested DNA from plaque-purified, CsCl gradient-isolated phages H-19J and 933J. The size of the intact DNA from each of the bacteriophages was estimated as 50-55 kilobase pairs on the basis of the sum of the sizes of the individual restriction fragments. The sizes of the fragments were determined by their relative electrophoretic mobilities when compared to Hind III reference fragments of DNA from coliphage.

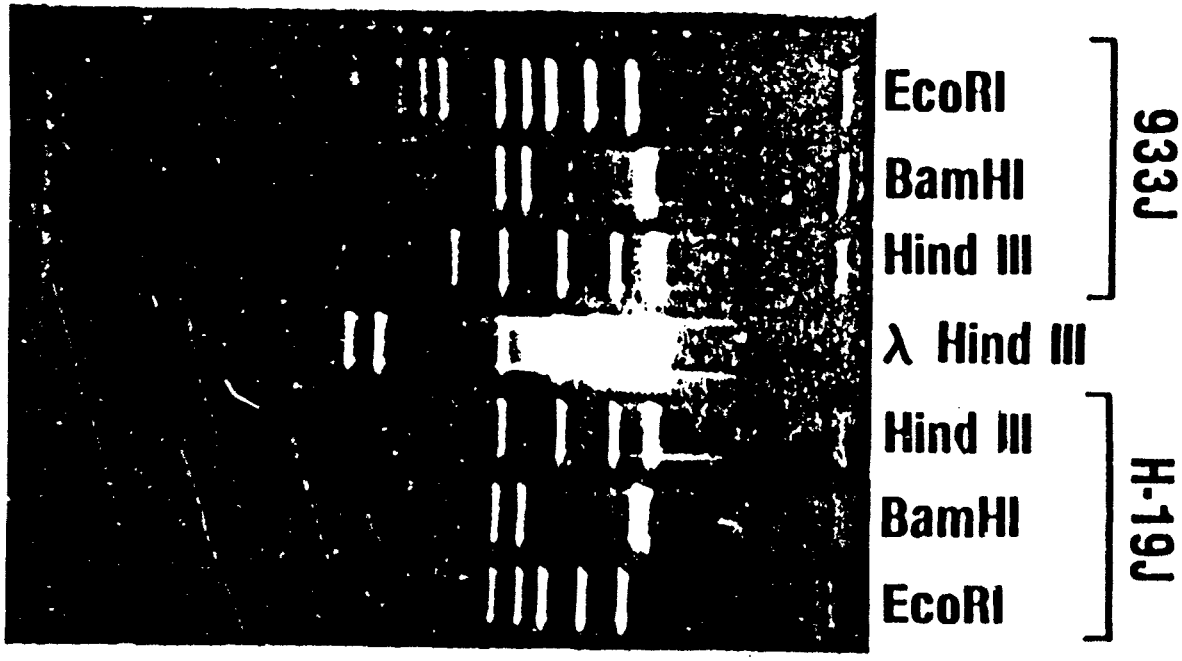


Figure 9. Agarose gel electrophoresis of ethidium bromide-stained restriction endonuclease-digested DNA from phage H-19J and phage 933J (O'Brien et al., 1984).

Table 3. SIZE ESTIMATES FOR RESTRICTION FRAGMENTS OF PHAGE 933J DNA

FRAGMENT NUMBER	FRAGMENT SIZE (KB)		
	ECORI	HIND III	BAMHI
1	13.5	23.0	17.0
2	8.0	12.5	(17.0)*
3	5.9	7.2	7.0
4	5.8	4.6	5.0
5	5.0	3.4	4.2
6	4.2	0.7	2.0
7	3.1	0.5	1.4
8	2.8		0.7
9	2.4		
10	2.0		
11	1.0		
12	0.8		
TOTAL**	54.5	51.9	54.4*

* There appear to be two different Bam HI fragments of approximately 17 kB in size. The 54.4 kB sum for the Bam HI fragments is based on this assumption.

**The estimated size of the phage genome based on the sum of the fragment sizes is 50-55 kB.

Table 5.

Radiolabeled phage H-19J DNA was used to probe Eco RI digests of chromosomal DNA prepared from a variety of strains known to produce Shiga-like toxin. These hybridizations were done at 65°C in an aqueous system in an attempt to detect additional phage related sequences present in these strains. A complete pattern of homology was observed between the H-19J probe, and 933J or H-19J phage DNA. Homologous bands were detected in the highly toxinogenic strain B821/2/1, but not in shigella or vibrio strains that make Shiga-like toxin. Several bands exhibiting phage homology were found in the E. coli normal flora #4 strain. H-19J DNA homology to the Q (late gene regulation) region of lambda was also detected, but the significance of this is unknown.

Table 5. SOUTHERN HYBRIDIZATIONS WITH WHOLE-PHAGE DNA

Eco Ri-digested Chromosomal DNA		H-19J Probe
A)	933J phage	+ 12 bands
B)	*V. parahaemolyticus*	-
C)	*V. cholerae* GN6300	-
D)	*V. cholerae* JBK70	-
E)	*E. coli* B821/2/1	+ 7 bands
F)	pBR328 plasmid	-
G)	*V. cholerae* 1074-78	-
H)	*V. parahaemolyticus* WP1	-
I)	*E. coli* K-12:395-1	-
J)	*E. coli* normal flora #4	+ 5 bands
K)	Lambda phage	+ 2 bands
L)	*S. dysenteriae* 60R	-
M)	*E. coli* normal flora #3	-
N)	*S. flexneri* M4243	-
O)	*S. dysenteriae*3818T	-
P)	H-19J phage	+ 12 bands

Relationships Among Phages 933J and H-19J to Phages H-19A, H-19B, and 933W

Further studies were performed to determine if any relationships existed between phages 933J and H-19J as compared to the independently isolated phages H-19A, H-19B, and 933W (Smith, 1983). These phages are representative isolates from Escherichia coli strains 933 and H-19 that were collected on different occasions by methods previously discussed. Studies were conducted to determine the specificity of lysogenic immunity conferred by these phages (Table 4). Diluted phage stocks were incubated with an Escherichia coli C600 lysogen containing independent phage isolates, plated in a soft overlay agar, and the formation of plaques was recorded. Escherichia coli C600 was used as the positive control in these lysogenic immunity studies. H-19A and H-19J are independent isolates of the same phage, whereas H-19B appears to be a different phage. The restriction of plaquing of phage 933J, but not phage H-19A/J, on lawns of C600(933W) indicates that phage 933J and phage H-19J are not identical. A further difference suggesting that phage 933J is not identical to phages H-19A/J is that 933J lysates are unstable (four-fold decrease in titre) when stored at $4^{o}C$ for three days. Furthermore, all of the phages except phage 933W were stable when heated at $58^{o}C$ for 30 minutes; the titre of 933W was decreased 1000-fold under these conditions. Escherichia coli strain 933 appears to possess two different Shiga-like toxin-converting phages, represented by isolates 933J and 933W.

Table 4.

Phage stocks were diluted 10^{-2} and plated in a soft agar overlay. The designations C600(H-19J), C600(H-19A), C600(933J), C600(H-19B), and C600(933W) indicate E. coli K-12 substrain c600 lysogenized with the phage H-19J, H-19A, 933J, H-19B, or 933W, respectively. Symbols: (+) formation of a large number (>10^4) of plaques on the host lawn after overnight incubation, (-) no plaques, and (*) less than 50 plaques. Phage samples were isolated with ultraviolet-light induction, and were then plaque-purified and subjected to centrifugation through CsCl step-gradients.

Host strain of *E. coli*	H-19J	H-19A	933J	H-19B	933W
c600	+	+	+	+	+
c600(H-19J)	-	-	-	-	+
c600(H-19A)	-	-	-	-	+
c600(933J)	-	-	-	-	+
c600(H-19B)	+	+	+	-	+
c600(933W)	+	+	*	+	-

Table 4. Capacity of different plaque-purified Shiga-like toxin-converting phages to form plaques on various *E. coli* hosts.

Isolation of Toxin-Converting Genes from Phage 933J DNA

A partial map of restriction endonuclease cleavage sites in phage 933J DNA was constructed using the restriction endonucleases Hind III, Eco RI, Bam HI, and Sal I (Figure 10). The circular map is shown in linear form and is cleaved between Hind III fragments 1 and 2. The partial map contains 12 Eco RI sites, 7 Hind III sites, 5 Bam HI sites, and 2 Sal I sites. Eleven of the Eco RI fragments were cloned into the plasmid vector pBR328, and then transformed into Escherichia coli strain HB101 under P4 + EK1 conditions. Highly toxinogenic clones were identified by a HeLa cell cytotoxicity assay. Eco RI fragment 2 was the only fragment consistently found to be present in all highly toxinogenic clones.

Hybrid plasmids containing portions of the Eco RI fragment 2 from phage 933J DNA were constructed by insertion into pBR328. The restriction endonuclease sites of the inserts of 9 hybrid plasmids (pJN20-pJN28) in addition to the toxinogenicity of each hybrid plasmid are shown in Figure 11. Results from subcloning demonstrated that sequences could be removed from each end of the Eco RI insert from pJN20 without inactivating the toxin-converting gene(s). The only subclones that were toxinogenic were those containing the region of DNA flanking the leftmost Hind III restriction site. Sonic lysates of HB101 (pJN25) contained a seven-fold increase in toxin (5×10^8 CD_{50} per milligram of protein in cell lysates) over those of the Escherichia coli 933 parental strains. Hence, the Shiga-like toxin-converting phage 933J does contain the Shiga-like toxin-converting gene(s).

Figure 10.

The circular map is displayed in a linear fashion and is arbitrarily broken between Hind III fragments 1 and 2. The 12 Eco RI sites, 7 Hind III sites, 5 Bam HI sites, and 2 Sal I sites are indicated. Eco RI or Hind III restriction fragments were ligated into pBR328, transformed into E. coli HB101, and highly toxinogenic clones were identified by a HeLa cell assay. Eco RI fragment 2 was the only common fragment observed to be present in all highly toxinogenic clones. The numbers below the map denote kilobase pairs and the arrows show the relative position of Eco RI fragment 2 within the linearized map.

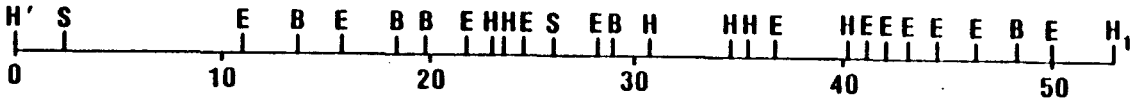


Figure 10. Map of restriction endonuclease cleavage sites in DNA from phage 933J.

Figure 11.

pJN20 through pJN28 are hybrid plasmids that contain portions of the Eco RI fragment 2 from phage 933J, inserted into pBR328. Restriction endonuclease sites are indicated for each insert. Toxigenicity of each plasmid is shown on the right. Only subclones containing the region of DNA flanking the leftmost Hind III restriction site were toxinogenic. Levels of toxin produced by pJN25 in Escherichia coli HB101 were seven-fold higher than those of the Escherichia coli 933 parental strain. The symbols refer to the restriction endonuclease enzymes as follows: E - Eco RI, Pl - Pst I, N - NcoI, P - Pvu II, B - Bam HI, H - Hind III, EV - Eco RV, C - Cla I, and S - Sal I. The bar scale located at the bottom of the figure represents 1 kilobase.

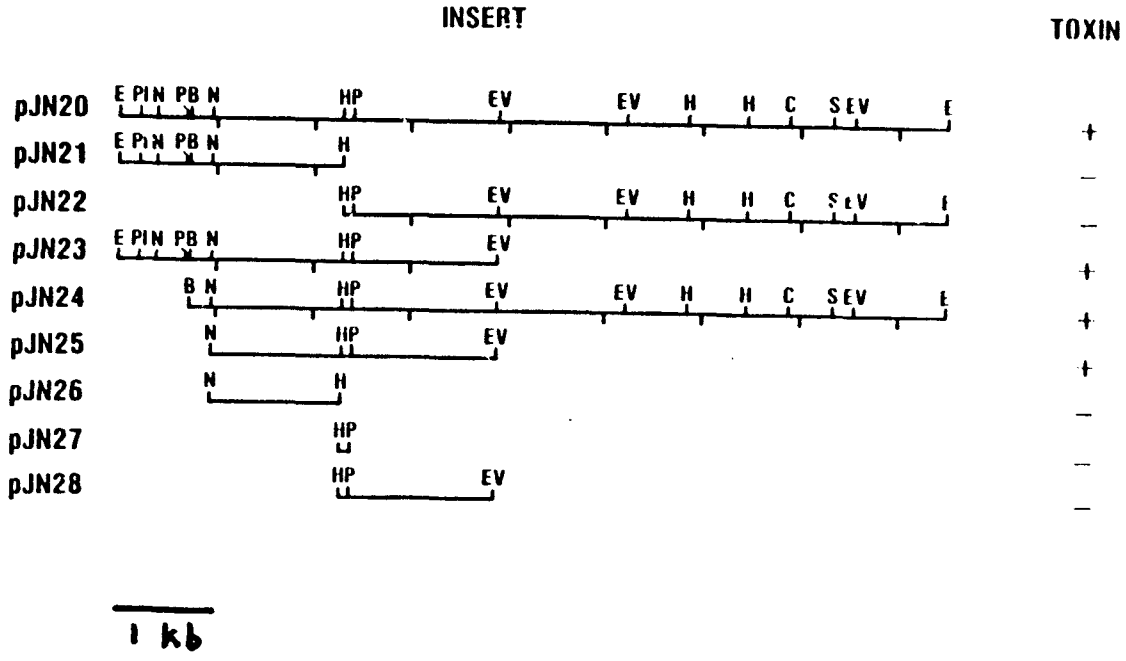


Figure 11. Toxigenicity of selected hybrid plasmids.

Analysis of Hybrid Plasmid-Encoded Proteins

Studies were conducted to analyze the polypeptides synthesized in minicells of Escherichia coli strain DS410, into which distinct hybrid plasmids were introduced. The hybrid plasmids pJN25, pJN26, pJN27, pJN28, and plasmid pBR328 were incorporated into minicells. The [^{35}S] methionine-labeled polypeptides synthesized in these minicells were fluorographed as shown in Figure 12. The highly toxinogenic subclone pJN25 (lane C) encoded two polypeptides of approximately 31.5 kD and 7kD. These two polypeptides are in close agreement with those previously reported for the A (31.5 kD) and B (7 kD) subunits of Shiga-like toxin. Subclone pJN26 (lane A), that contains the region of DNA that extends in a leftward direction from the Hind III restriction site, encodes only the 7 kD polypeptide (B subunit) but not the 31.5 kD polypeptide. In contrast, subclone pJN28 (lane B), that extends in a rightward direction from the Hind III restriction site, encodes a 20 kD polypeptide but not the 7 kD polypeptide. This 20 kD polypeptide (A') is most likely a truncated polypeptide that corresponds to a portion of the 31.5 kD A subunit from pJN25. Subclone pJN27 contains a small internal fragment of approximately 100 base pairs from the A subunit. Thus, the Shiga-like toxin-converting genes present on Escherichia coli phage 933J are structural genes that appear to encode a 31.5 kD polypeptide corresponding to the A subunit and a 7 kD polypeptide corresponding to the B subunit of Shiga-like toxin.

Figure 12.

The following plasmids were incorporated into minicells: A) pJN26, B) pJN28, C) pJN25, D) pJN27, and E) pBR328. Numbers on the left indicate the molecular mass in kilodaltons (kD) of selected polypeptide standards. Arrows on the right point to the polypeptides not encoded by the pBR328 cloning vector. The toxin-positive subclone pJN25 (lane C) encoded two polypeptides of approximately 31.5 kD and 7 kD that are in excellent agreement with those previously reported for the A and B subunits of Shiga-like toxin. Subclone pJN26 (lane A) contains the region of DNA extending leftward from the Hind III restriction site and encodes the 7kD (B subunit) polypeptide, while subclone pJN28 (lane B) extends rightward from the Hind III site and encodes a 20 kD polypeptide. This 20kD protein (A') is most likely a truncated polypeptide corresponding to a portion of the 31.5 kD A subunit from pJN25. Subclone pJN27 contains a small ($\leq$100 base pairs) internal fragment from the A subunit and is included as an additional control for identification of the ampicillin resistance proteins that are present in all the lanes. pBR328 (lane E) and pJN28 (lane B) also code for the 26 kD chloroamphenicol transacetylase polypeptide.

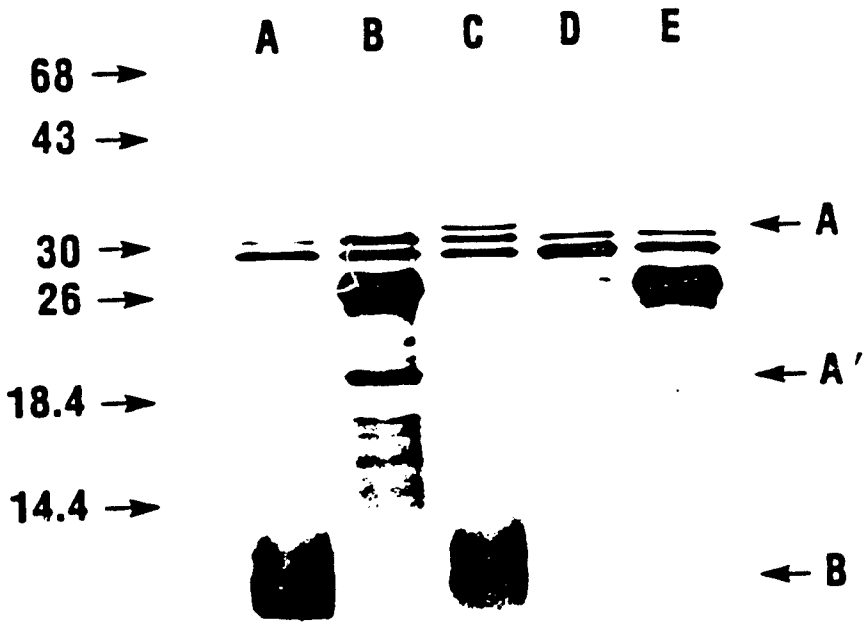


Figure 12. Autoradiograph of the [^{35}S] methionine-labeled polypeptides synthesized in minicells of E. coli strain DS-410 containing hybrid plasmids (Newland et al., 1985; submitted for publication to Science magazine).

Southern Hybridizations with Phage H-19J and pJN25 DNA

Radiolabeled phage H-19J DNA was used to probe Eco RI digested chromosomal DNA prepared from specific strains known to produce Shiga-like toxin. Hybridizations were conducted at 65°C in an aqueous system in order to detect phage-related sequences present in these chosen strains. Table 5 demonstrates that homology was observed between the H-19J probe, and 933J or H-19J phage DNA. The highly toxinogenic Escherichia coli strain 3821/2/1 exhibited homologous bands, but Shigella and Vibrio strains that produce Shiga-like toxin exhibited no detectable homology with labeled whole-phage DNA of the probe. Homologous bands were detected in Escherichia coli normal flora #4 strain, that produces only trace-levels of Shiga-like toxin. Phage H-19J DNA was also shown to be homologous to the Q region (late gene regulation) of lambda. Thus, H-19J phage-related sequences are contained in both high and low Shiga-like toxin producing Escherichia coli strains and phage lambda.

Additional hybridizations were conducted with radiolabeled pJN25 DNA. A pattern of homology was observed between the pJN25 probe and Shigella dysenteriae 1, Shigella flexneri, and phage 933J DNA as shown in Figure 13. Hence, the insert in pJN25 which contains the Shiga-like toxin structural genes as demonstrated by the minicell analysis, is homologous with chromosomal DNA sequences present in Shigella. It is very likely, therefore, that the sequences encoding the Shiga-like toxin genes in phage 933J are homologous with the structural genes for Shiga toxin in Shigella dysenteriae 1 and Shigella flexneri. Additional experiments are in progress to determine the nucleotide sequence of the cloned structural genes for Shiga-like toxin.

Figure 13.

Hybridizations were conducted at 65°C in an aqueous system. Lane A contains Eco RI-digested DNA from Shigella dysenteriae 1, lane B contains Eco RI-digested DNA from phage 933J, and lane C contains Eco RI-digested DNA from Shigella flexneri. The relative electrophoretic mobilities of the hybridized fragments in lane A and lane C are shown in comparison to the hybridized 8.5 kB Eco RI fragment #2 (from phage 933J DNA) in lane B.

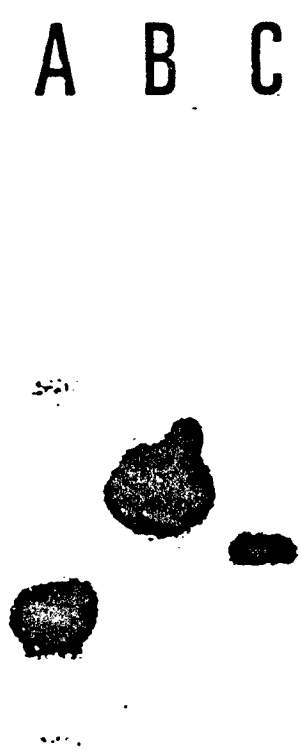


Figure 13. Autoradiograph of ^{32}P-labeled pJN25 DNA hybridized in Southern blots with Eco RI-digested DNA from S. dysenteriae 1, phage 933J, and S. flexneri.

DISCUSSION

At the time these studies were initiated, it had been shown that some Escherichia coli strains associated with diarrheal disease in man produced high levels of Shiga-like toxin but were not enteroinvasive nor were they able to make heat-labile or heat-stable toxins. It had also been established that one such strain, Escherichia coli 026 strain H-19, harbored two distinct Shiga-like toxin-converting phages. At that time, neither of the H-19 phages had been fully characterized. Moreover, no phage had been isolated from Escherichia coli 0157:H7 strain 933. A major goal of these studies was to isolate a Shiga-like toxin-converting phage from Escherichia coli strain 933, and to compare its morphology, structural polypeptide content, and DNA restriction fragment pattern with one of the previously isolated strain H-19 phages. This objective was achieved. Two phages were isolated from each Escherichia coli strain upon ultraviolet induction of each lysogen. Escherichia coli strain H-19 contained phage H-19A/J (independent isolates of the same phage) and phage H-19B, whereas Escherichia coli strain 933 contained phage 933J and phage 933W. Both H-19A/J and H-19B are converting phages that determine production of high titres of Shiga-like toxin. One of the phages contained in Escherichia coli strain 933 is also a Shiga-like toxin-converting phage (933J). The other phage (33W) makes a cytotoxin with the same biological activities of Shiga-like toxin but the toxin cannot be neutralized by antibody to purified Shiga toxin (N. Strockbine, L. Marques, and A. O'Brien, unpublished data).

Phage 933J and phage H-19A/J are indistinguishable, as judged by the following criteria: morphology, virion proteins, restriction endonuclease fragments of genomic DNA, DNA-DNA hybridization in Southern blots, and heat stability. However, the restriction of plaquing of phage 933J but not H-19A/J on lawns of c600(933W) indicates that they are not identical.

One possible explanation for the restriction of plaquing of phage 933J on lawns of c600(933W) is that phage 933J and 933W are similar. However, the immunity specificity pattern of phage 933W does not support this contention because phage 933W can plaque on lawns of c600(933J). Thus, phage 933J is closely related but not identical to phage H-19A/J, but is different from phage 933W. On the basis of lysogenic immunity patterns and resistance to thermal inactivation, the Shiga-like toxin-converting phage H-19B and the HeLa cell cytotoxin-converting phage 933W also differ both from each other and from phages H-19A/J and 933J.

A further aim of this project was to determine how the H-19A/J and 933J phages control the production of Shiga-like toxin. It had been demonstrated that Escherichia coli K-12 substrain 395-1 makes low levels of Shiga-like toxin. Thus, it appears that the genome of Escherichia coli K-12: 395-1 may contain the toxin structural gene or genes. However, lysogenization of Escherichia coli K-12: 395-1 results in an extraordinary increase (10,000-fold) in the quantity of Shiga-like toxin produced. Converting phages could possess either the Shiga-like toxin structural gene(s) or regulatory elements that act on toxin structural gene(s) already present in the host bacterium. To determine which of these possibilities was correct, it was necessary to isolate and

characterize the toxin-converting genes from phage DNA by recombinant DNA techniques.

To achieve this goal, we cloned and subsequently subcloned the Shiga-like toxin-converting genes of phage 933J and examined the products of the subclones by minicell analysis. We found that the highly toxinogenic subclone pJN25 encoded two polypeptides of approximately 31.5 kD and 7 kD. The sizes of these two polypeptides are in close agreement with those previously reported for the A (31.5 kD) and B (7 kD) subunits of Shiga-like toxin. Moreover, subclone pJN28 which extends in a rightward direction from the Hind III restriction site encoded a 20 kD polypeptide. This 20 kD polypeptide (A') is most likely a truncated polypeptide that corresponds to a portion of the 31.5 kD subunit from pJN25. The minicell data support the theory that phage 933J DNA contains the Shiga-like toxin structural genes. The findings also lend credence to the idea that the gene for the B subunit is located to the left of the gene coding for the subunit of Shiga-like toxin with respect to the physical maps shown in Figure 11. It is not known at the present time if these structural genes are contiguous. The 20 kD polypeptide produced by pJN28 leads us to hypothesize that transcription/translation of these genes proceeds from a right to a leftward direction. This model representing the arrangement of the Shiga-like toxin structural genes is consistent with all of the results generated to date. Further studies are required to confirm this model of the organization of the structural genes for high-level production of Shiga-like toxin in Escherichia coli.

Previous studies established the facts that a family of Verotoxin (i.e., Shiga-like toxin)-converting phages exists in Escherichia

coli throughout nature (Smith et al., 1983). The distribution of the phages of this family and the degree of relatedness among these phages have not been determined. However, in this study it was observed that ^{32}P-labeled whole-phage DNA from phage H-19A/J hybridized in Southern blots with specific restriction fragments of genomic DNA from selected strains of Escherichia coli 026, 0111, and 0145 have been associated with diarrheal disease in humans. However, it has also been shown that certain low Shiga-like toxin producing Escherichia coli strains contain bacteriophages. To determine the relationship among the family of Shiga-like toxin-converting phages, specific phage DNA probes in addition to Shiga-like toxin structural gene probes must be constructed. The availability of such probes would allow a study of the distribution of each member of the phage family in Escherichia coli strains in nature.

Additionally, various Shiga-like toxin-producing strains were probed for homology with a Shiga-like toxin structural gene probe from the hybrid plasmid pJN25. The ^{32}P-labeled DNA from pJN25 hybridized in Southern blots with specific restriction fragments of genomic DNA from high and low Shiga-like toxin producing Escherichia coli strains, Shigella dysenteriae 1, and Shigella flexneri. These findings suggest that genes for Shiga-like toxin production are also present in various strains of Escherichia coli, in addition to Shigella dysenteriae, and Shigella flexneri.

Whatever the specific organization of Shiga-like toxin structural genes, a strong correlation exists between the high level production of Shiga-like toxin and the ability of Escherichia coli to produce hemorrhagic colitis in humans (O'Brien et al., 1983a). The

presence of converting phages in such clinical isolates of Escherichia coli and the presence of the Shiga-like toxin structural genes in these phages is also correlated with the ability of the converting phages to determine high levels of toxin production in Escherichia coli K-12 substrain 395-1. The studies described in this report (O'Brien et al., 1984; Newland et al., 1985, submitted for publication) are but a first step in the investigation of the molecular biology of Shiga-like toxin. Such an investigation should ultimately lead to a better understanding of the role of Shiga-like toxin in the pathogenesis of gastrointestinal infections caused by Escherichia coli.

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